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Cyclin D1 Overexpression Is a Critical Event in Gallbladder Carcinogenesis and Independently Predicts Decreased Survival for Patients with Gallbladder Carcinoma¹

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ABSTRACT

This study was designed to test the hypothesis that cyclin D1 overexpression is involved in the multistep process of gallbladder carcinogenesis and can be used to predict poor prognosis for patients with gallbladder carcinoma (GBC). Cyclin D1 expression was examined immunohistochemically in a series of specimens, including 8 normal epithelia, 8 benign adenomyoma lesions, 6 precancerous adenomas, and 37 carcinomas of the gallbladder. Four of the 6 (67%) adenomas and 15 of the 37 (41%) adenocarcinomas demonstrated cyclin D1 overexpression (>5% nuclear staining), whereas all normal epithelia and adenomyoma lesions were negative for cyclin D1. Kaplan-Meier curves showed that cyclin D1 overexpression was significantly related to decreased overall survival ($P < 0.05$) in patients with GBCs. The Cox proportional hazards model identified cyclin D1 overexpression as an independent prognostic marker for death ($P = 0.024$; risk ratio, 4.2; 95% confidence interval, 1.2-14.7). To test whether cyclin D1 overexpression is a critical event in gallbladder neoplasms, cyclin-dependent kinase inhibitor p27^{Kip1} was introduced to ascertain how cyclin D1 affects clinical outcomes. Subsequently, neoplasms were divided into three groups on the basis of the combination of cyclin D1 expression and p27^{Kip1} status, which had been determined previously. Group 1 showed no abnormality in either cyclin D1 or p27^{Kip1} expression. Group 2 showed aberrant expression of one of the two proteins,

whereas group 3 showed concurrent abnormalities in both proteins. Results indicated that overall survival was greatest in group 1, followed by a significant decrease in group 2 and a more precipitous decrease in group 3. In conclusion, cyclin D1 overexpression is an early event in gallbladder carcinogenesis and independently predicts decreased survival for patients with GBC.

INTRODUCTION

Multiple genetic or epigenetic changes contribute to the multistep process of human carcinogenesis, and some of these changes help with the monitoring of this multistep process. It is important to understand the carcinogenic process and its corresponding molecular basis for each type of cancer. For gallbladder carcinogenesis, the dysplasia to carcinoma and adenoma to carcinoma conventional progressions have been generally ascertained (1, 2). However, the molecular mechanism underlying the development and progression of GBC³ is not fully understood. This is possibly because of the relative rarity of GBC, which accounts for only 3% of gastrointestinal cancers and 0.5% of all human malignancies, limiting the number of tumor samples available for study. GBC is one of the most biologically virulent cancers and is difficult to cure by conventional procedures. Elucidation of its molecular basis may be helpful in developing and identifying prognostic biomarkers.

Escaping from normal cell cycle controls is critical for the various processes of carcinogenesis (3-6). Cell cycle regulators more often altered in cancers are those controlling G₁-S-phase progression. G₁-S transition is controlled through interaction of several types of molecules, including CDKs, their positive regulators, cyclins (cyclins D1, D2, D3, and E), and their negative regulators, CDK inhibitors and retinoblastoma protein. CDK inhibitors fall into two classes on the basis of their sequence homology: (a) the INK family, which includes p16^{INK4A}, p15^{INK4B}, p18^{INK4A}, and p19^{INK4B}; and (b) the Cip/Kip family, which includes p21^{WAF1/Cip1}, p27^{Kip1}, and p57^{Kip2}. Among these proteins controlling the G₁-S transition, cyclin D1 is one of the most strongly implicated in tumorigenesis. Cyclin D1 exerts its effects on cell cycle progression via two mechanisms: (a) cyclin D1-CDK complexes inactivate retinoblastoma protein by phosphorylation; and (b) these complexes bind and sequester Cip/Kip proteins stoichiometrically (7). Evidence for the oncogenic potential of cyclin D1 is provided by studies with numerous models, in which elevated expression of cyclin D1 shortens the G₁ phase of the cell cycle and enhances malignant transformation (8-12).

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³ The abbreviations used are: GBC, gallbladder carcinoma; CDK, cyclin-dependent kinase; TNM, tumor-node-metastasis.

Cyclin D1 overexpression, either with or without gene amplification, has been shown in a variety of human malignancies, including breast (13–18), colon (19), lung (20), esophageal (21–23), liver (24–26), pancreatic (27), tongue (28), oral verrucous (29), pharyngeal (30), and laryngeal (31) cancers and has been identified as a prognostic biomarker for breast (16), lung (20), esophageal (22, 23), pancreatic (27), tongue (28), pharyngeal (30), and laryngeal (31) cancers. The role of cyclin D1 in GBC has not been extensively studied. Recently, we showed that cyclin D1 overexpression is significantly associated with poor clinical outcomes for patients with extrahepatic bile duct cancer (32), another type of biliary tract malignancy. Moreover, cyclin D1 overexpression has been suggested to be an early event in human breast (18), lung (33), and colon (34, 35) carcinogenesis and in mouse skin carcinogenesis (36). On the basis of this background, we postulated that cyclin D1 overexpression may be involved in the multistep process of gallbladder carcinogenesis and may be useful as a prognostic marker for GBC. To test this hypothesis, we attempted to study cyclin D1 expression using immunohistochemistry in a series of surgically resected specimens, including normal epithelium, benign lesion adenomyoma, adenoma, and adenocarcinoma of gallbladder from patients treated surgically who had been in a postoperative follow-up phase.

MATERIALS AND METHODS

Patients. Thirty-seven GBC lesions surgically resected from 36 patients (one patient had two cancers) between January 1990 and April 1999 in our department were studied. There were 17 males and 19 females, with a median age of 65 years (age range, 45–84 years). The clinicopathological variables were evaluated following the General Rules for Surgical and Pathological Studies on Cancer of Biliary Tract of the Japanese Society of Biliary Surgery (37). Cancers were staged following the TNM system of the International Union against Cancer (38). The clinicopathological features are described in Table 1. Surgical procedures included 23 cholecystectomies (single cholecystectomy or cholecystectomy with resection of <2 cm depth of the liver bed) and 13 extended operations (resection of adjacent organs in addition to the gallbladder). Four patients who underwent palliative surgery were excluded from survival analysis.

The status of p27^{Kip1} expression in this group of GBCs has been studied previously (39). The 37 tumors were classified as 16 tumors with decreased p27^{Kip1} expression (<50% of tumor cells showing nuclear p27^{Kip1} immunoreactivity), and 21 tumors with normal p27^{Kip1} expression (≥50% of tumor cells showing nuclear p27^{Kip1} immunoreactivity).

Six surgically resected specimens of gallbladder adenomas were studied. None of the adenomas had foci of carcinoma *in situ*. Eight specimens of adenomyoma, as well as eight specimens of normal gallbladder epithelia obtained from healthy persons who had undergone cholecystectomy when donating partial livers for transplantation, were also included in this study.

Immunohistochemistry. Five-μm-thick sections were cut from formalin-fixed paraffin-embedded tissue blocks and mounted on gelatin-coated glass slides. The sections were de-

Table 1 Clinicopathological features and Cyclin D1 expression in gallbladder carcinomas

Factors	Total no.	No. overexpressing cyclin D1 (%) ^a
Age (yr)		
≤65	20	8 (40)
>65	17	7 (41)
Gender		
Male	17	8 (47)
Female	20	7 (35)
Histological type		
PA ^b	12	5 (42)
WDTA	12	6 (50)
MDTA	6	2 (33)
PDTA	3	2 (67)
Others ^c	4	0
Vascular invasion		
Yes	12	5 (42)
No	25	10 (40)
Lymphatic invasion		
Yes	12	5 (42)
No	25	10 (40)
Perineural invasion		
Yes	13	4 (31)
No	24	11 (46)
Lymph node involvement		
Yes	12	3 (25)
No	25	12 (48)
Stage		
I	11	7 (64)
II	11	4 (36)
III	7	1 (14)
IV	8	3 (38)
p27 ^{Kip1} expression		
<50%	16	7 (44)
≥50%	21	8 (38)
Surgical procedure		
Cholecystectomy ^d	24	14 (58)
Extended operation ^e	13	8 (62)

^a No comparisons reached the significant level (χ^2 test).

^b PA, papillary adenocarcinoma; WDTA, well-differentiated tubular adenocarcinoma; MDTA, moderately differentiated tubular adenocarcinoma; PDTA, poorly differentiated tubular adenocarcinoma.

^c Includes one mucinous adenocarcinoma, one signet ring cell carcinoma, one adenosquamous cell carcinoma, and one undifferentiated carcinoma.

^d Single cholecystectomy or cholecystectomy with resection of less than 2 cm depth of the liver bed.

^e Resection of adjacent organs in addition to the gallbladder.

waxed in xylene, rehydrated through graded concentrations of ethanol, and then treated with 0.5% hydrogen peroxide at room temperature for 30 min to block endogenous peroxidase activity. The sections were immersed in 10 mM citrate buffer (pH 6.0) and processed in an autoclave for 10 min at 120°C for antigen retrieval (32, 40). Anti-cyclin D1 antibody (Clone DCS-6; NeoMarkers, Union City, CA; dilution, 1:50) was applied, and incubations were performed overnight at 4°C. Immunohistochemistry was performed by the avidin-biotin complex technique using the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA) with 3,3'-diaminobenzidine tetrahydrochloride development and hematoxylin counterstaining. Negative controls were sections stained without the primary antibody. Nuclear staining was considered to indicate specific cyclin D1 immunoreactivity.

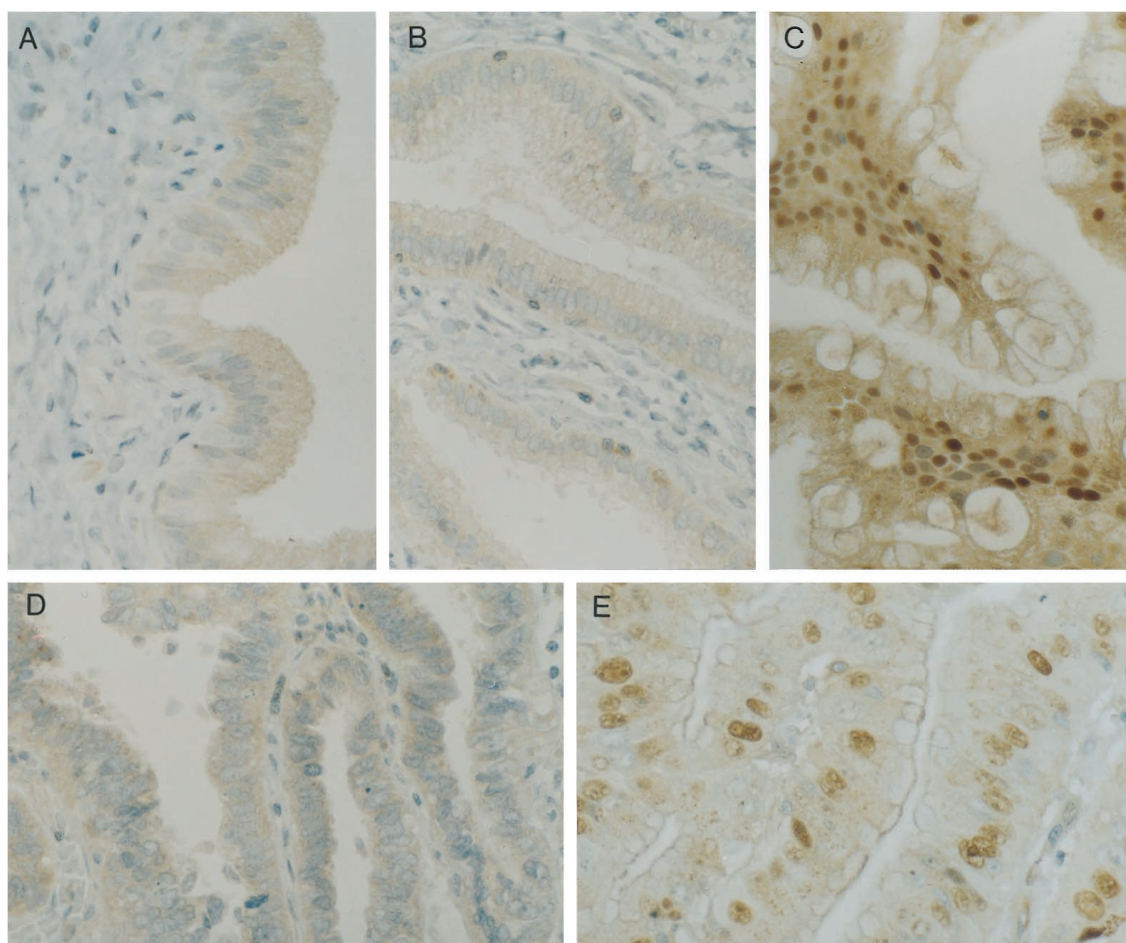


Fig. 1 Examples of cyclin D1 immunohistochemical staining. Normal gallbladder epithelia (A) and an adenomyoma (B), both of which were cyclin D1 negative. C, an adenoma demonstrating cyclin D1 overexpression. D, a cyclin D1-negative carcinoma. E, a carcinoma demonstrating cyclin D1 overexpression ($\times 350$).

For each case, one section having the greatest area of tumor and one section containing noncancerous gallbladder epithelium were selected for immunohistochemical staining from the tissue blocks on the basis of observation of H&E-stained sections. The scoring of cyclin D1 immunoreactivity was based on examination of 10 high-power ($\times 400$) microscopic fields or total tumor (when tumor was smaller than 10 fields) for each case. According to a widely accepted criterion (17, 18, 23, 26, 30–32), tumors that expressed strong immunoreactivity in $>5\%$ of the cells were considered cyclin D1 positive (overexpression). All slides were interpreted independently by two investigators (A-M. H. and X. L.) without knowledge of the patients' clinical courses.

Statistical Analysis. The associations between cyclin D1 expression and clinicopathological variables were assessed using the χ^2 test. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test (41). The significance of various parameters for survival was analyzed by the Cox proportional hazards model (42). $P < 0.05$ was considered statistically significant.

RESULTS

Normal gallbladder epithelia from either healthy persons or GBC patients were consistently negative with regard to cyclin D1 nuclear immunoreactivity (Fig. 1A), demonstrating $<1\%$ positive cells. All eight adenomyoma lesions were also cyclin D1 negative (Fig. 1B). For seven of these specimens, the percentage of positive epithelial cells was $<1\%$, and in the eighth specimen cyclin D1 immunoreactivity was observed in 3% of the epithelial cells. Four of the six (67%) adenomas showed cyclin D1 overexpression ($>5\%$ positive cells; Fig. 1C) with the percentage of positive cells ranging from 8% to 90%.

Twenty-two of the 37 GBCs were cyclin D1 negative (59%; Fig. 1D), and the remaining 15 were cyclin D1 positive (41%; Fig. 1E). For the 15 cyclin D1-positive cancers, the percentage of cyclin D1-positive cells ranged from 6% to 90%. Cyclin D1 expression was not related to any clinicopathological parameters such as age, tumor histological type, lymphatic metastasis, TNM stage, p27^{Kip1} expression status, or surgical procedure (Table 1).

The duration of follow-up of the patients ranged from

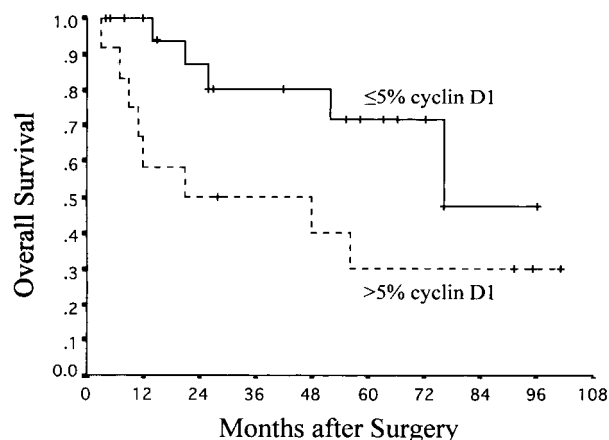


Fig. 2 Kaplan-Meier curves for overall survival according to cyclin D1 expression in 32 patients who underwent radical surgery for GBC. Tick marks indicate surviving patients and the duration of follow-up. $P < 0.05$, log-rank test.

3–101 months (median follow-up, 28 months). The Kaplan-Meier method and log-rank test were used to evaluate the effect of cyclin D1 expression on postoperative survival in 32 patients with GBCs, excluding four patients who underwent nonradical surgery. The 3-year and 5-year overall survival rates were 68% and 53% for all of the 32 patients. The corresponding figures were 80% and 71% for the cyclin D1-negative group and 50% and 30% for the cyclin D1-positive group, respectively. Cyclin D1 overexpression significantly predicted reduced overall survival (Fig. 2; $P < 0.05$). Multivariate analysis by the Cox proportional hazards model identified cyclin D1 overexpression ($P = 0.024$; risk ratio, 4.2; 95% confidence interval, 1.2–14.7), decreased p27^{Kip1} expression ($P = 0.024$; risk ratio, 4.2; 95% confidence interval, 1.3–13.7), and TNM stage III/IV ($P = 0.001$; risk ratio, 5.9; 95% confidence interval, 1.5–23.0) as independent predictors of death. The 3-year and 5-year overall survival rates were 84% and 71% for the patients who underwent cholecystectomy and 40% and 0% for those treated by extended operations. There was a significant difference between the survival curves ($P = 0.007$, log-rank test); however, the surgical procedure had no significance as an independent prognostic factor in the Cox proportional hazards model analysis. The effect of surgical procedure on survival probably resulted from the influence of disease stage; indeed, 9 of the 13 patients who underwent extended operations had stage III or stage IV tumors.

Drawing together the data of our present study and the previous one (39), in which $<50\%$ p27^{Kip1} nuclear staining was defined as decreased p27^{Kip1} expression, GBCs were classified into three groups on the basis of cyclin D1 and p27^{Kip1} expression: (a) group 1, no abnormality in either cyclin D1 or p27^{Kip1} expression ($\leq 5\%$ cyclin D1 and $\geq 50\%$ p27^{Kip1}); (b) group 2, altered expression of one of the two proteins ($>5\%$ cyclin D1 and $\geq 50\%$ p27^{Kip1} or $\leq 5\%$ cyclin D1 and $<50\%$ p27^{Kip1}); and (c) group 3, concurrent abnormalities in both proteins ($>5\%$ cyclin D1 and $<50\%$ p27^{Kip1}). Kaplan-Meier curves showed that overall survival was significantly shorter in group 2 ($n =$

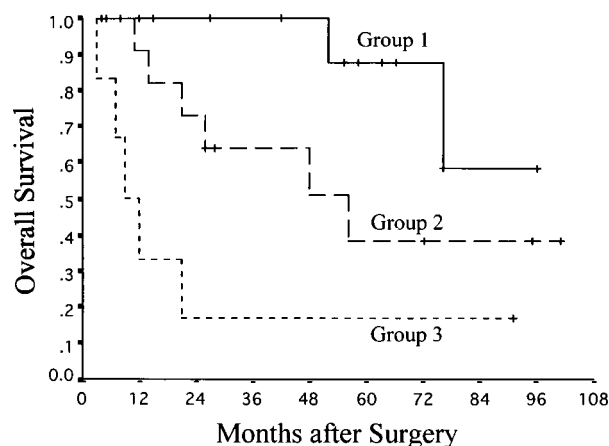


Fig. 3 Kaplan-Meier curves for overall survival according to the combination of cyclin D1 and p27^{Kip1} expression in 32 patients who underwent radical surgery for GBC. Tick marks indicate surviving patients and the duration of follow-up. Group 1, no abnormality in either cyclin D1 or p27^{Kip1} expression ($\leq 5\%$ cyclin D1 and $\geq 50\%$ p27^{Kip1}); group 2, aberrant expression of one of the two proteins ($>5\%$ cyclin D1 and $\geq 50\%$ p27^{Kip1} or $\leq 5\%$ cyclin D1 and $<50\%$ p27^{Kip1}); group 3, coincident abnormalities in the expression of both proteins ($>5\%$ cyclin D1 and $<50\%$ p27^{Kip1}). Group 1 versus group 2, $P < 0.05$; group 2 versus group 3, $P < 0.05$; group 1 versus group 3, $P < 0.01$ (log-rank test).

14) than in group 1 ($n = 12$) and was even shorter in group 3 [$n = 6$; Fig. 3; group 1 versus group 2, $P < 0.05$; group 2 versus group 3, $P < 0.05$; group 1 versus group 3, $P < 0.01$ (log-rank test)]. The 3-year and 5-year overall survival rates were 100% and 88% for group 1, 64% and 38% for group 2, and 17% and 17% for group 3.

DISCUSSION

The involvement of cyclin D1 overexpression has been reported previously in a wide range of human cancers. To determine the potential oncogenic role of cyclin D1 in human gallbladder carcinogenesis, we designed the present study to evaluate the levels of cyclin D1 expression in a series of surgically resected specimens including normal epithelia, adenomyoma lesions, precancerous adenomas, and adenocarcinomas of the gallbladder. Cyclin D1 overexpression was frequently observed in adenocarcinomas and even in adenomas, but not in any specimen of normal epithelium or adenomyoma. This strongly suggests that increased cyclin D1 expression is an early event in gallbladder carcinogenesis, probably playing a critical role in the transformation of gallbladder epithelial cells. Recently, cyclin D1 overexpression has also been observed in precursor lesions for other types of cancers, including breast hyperplasia (18), atypical adenomatous hyperplasia of the lung (33), and adenomatous polyps of the colon (34, 35).

It is interesting to note that, in this study, the frequency of cyclin D1 overexpression was higher in adenomas (67%) than in cancers (41%). There are two possible interpretations for this novel phenomenon: (a) in the process of gallbladder carcinogenesis, cyclin D1 overexpression may only be needed for establishment of the transformed phenotype, and in some cases it may no longer be needed for maintenance of the transformed

phenotype once the lesions have become malignant; and (b) the dysplasia to carcinoma sequence, as well as the adenoma to carcinoma progression, and other routes also contribute to the development of GBCs. Overexpression of cyclin D1 may be involved only in the adenoma to carcinoma route of gallbladder carcinogenesis and may not be involved in other pathways.

The most important finding in this study is that accumulation of cyclin D1 adversely affects clinical outcome and serves as an independent marker predicting decreased survival for patients with GBC. This is easy to understand, because cyclin D1 shortens the G₁ phase and accelerates the cell cycle (8). We have recently found that deregulation of p27^{Kip1}, another key protein in G₁-S transition control, significantly correlates with poor prognosis for GBC patients (39). To ascertain how cyclin D1 affects clinical outcome, p27^{Kip1} was introduced into this study. The postoperative survival rate was greatest in patients with no abnormality in either cyclin D1 or p27^{Kip1} expression, followed by a significant decrease in those with aberrant expression of one of the two proteins, and was even lower in those with concurrent abnormalities in both proteins (Fig. 3). These data suggest that cyclin D1 overexpression, together with reduced p27^{Kip1} expression, affects the clinical course of GBC.

In conclusion, cyclin D1 overexpression is an early event in gallbladder carcinogenesis and independently predicts poor prognosis for patients with resectable GBC.

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